Characterization of the tripartite drug efflux pumps of *Porphyromonas gingivalis* ATCC 33277

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SUMMARY

The periodontal pathogen, *Porphyromonas gingivalis* ATCC 33277 has six gene clusters that encode tripartite drug efflux pumps. To examine the effects of the drug efflux pumps on its antibiotic sensitivity, six mutants were constructed, each defective in the membrane fusion protein gene of each gene cluster. Compared to the wild-type strain, all mutants exhibited an elevated sensitivity to tetracycline, and two mutants with deletions in the PGN_1431 and PGN_1680 genes showed an increased sensitivity to various types of antibiotics. These results suggest that the activity of drug efflux systems may affect antibiotic sensitivity in *P. gingivalis*.

KEY WORDS: Porphyromonas gingivalis, Drug efflux pump, Antibiotic sensitivity, Membrane fusion protein, Phylogenetic tree.

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Antibiotics are indispensable for treating many infectious diseases caused by various bacterial pathogens. However, excessive use of antibiotics is known to result in an increase in strains of antibiotic-resistant bacteria. Recent studies have revealed that overexpression of drug efflux pumps is one of the reasons underlying the reduced antibiotic sensitivity of various bacteria (Piddock, 2006). To date, drug efflux pumps have been found in a wide variety of gram-positive and gram-negative bacteria, and are currently classified into five families (Sun *et al*., 2014). Drug efflux pumps in the RND family are composed of an outer membrane protein (OMP), a cytoplasmic membrane protein (CMP), and a periplasmic protein termed the membrane fusion protein (MFP). Drug efflux pumps in the ABC family are composed of CMPs with an ATP-binding domain and a permease domain, but pumps in this family often function by coupling with OMP and MFP. Both the RND-family pumps and the ABC-family pumps with OMP and MFP are known as tripartite drug efflux pumps (TDEPs).

Porphyromonas gingivalis is a gram-negative anaerobic pathogen that causes periodontitis (Bostanci and Belibasakis, 2012). Dentists often prescribe antibiotics to treat progressive periodontitis. Recent genome projects on *P. gingivalis* have determined the complete nucleotide sequences of three strains (Nelson *et al*., 2003; Naito *et al*., 2008; Watanabe *et al*., 2011). Analysis of the genome of *P. gingivalis* ATCC 33277 has revealed that this strain possesses six

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gene clusters encoding TDEPs. One of these is the *xep* system, which was previously reported by Ikeda and Yoshimura (2002). The *xep* system was found to be involved in efflux of multiple antibiotics. The present study constructed six mutants, each deficient in the MFP gene present in each gene cluster, and compared the antibiotic sensitivity of these mutants to that of the wild-type strain.

P. gingivalis ATCC 33277 was used as the wild-type strain. Six mutants, each deficient in the gene encoding a periplasmic MFP, were constructed as follows. The regions upstream and downstream of each target gene were amplified by PCR using the primers shown in Table 1. The PCR products were digested with appropriate restriction enzymes and ligated to a pUC19 vector, resulting in pUC-MFP plasmids. The pUC-MFPs were digested with *Bam*HI or *Bgl*II, and an erythromycin resistance cassette (*ermFAM*) (Kikuchi *et al*., 2009) was inserted at the *Bam*HI or *Bgl*II site in the same direction as the original gene, producing pUC-MFP::*ermFAM* plasmids. The nucleotide sequences upstream and downstream of the target genes in these plasmids were verified. Then, the pUC-MFP::*ermFAM* plasmids were linearized with *Pvu*II and introduced by electroporation into the wild-type strain, and the MFP gene-deletion mutants were selected on enriched brain heart infusion (BHI) blood agar (3.7% BHI broth, 0.5% yeast extract, 0.1% cysteine-HCl, 5% sheep defibrinated blood and 1.5% agar) containing erythromycin at 10 μg/mL. These mutants were verified by PCR. Double-knockout mutants deficient in both PGN_1431 and PGN_1680 genes were constructed by introducing a cefoxitin resistance cassette (*cfxA*) (Ichimura *et al*., 2010) at the PGN_1431 locus of MFD1680, as described above. Bacterial strains were subcultured anaerobically at 37°C on PG agar (4% tryptic soy agar, 0.5% BHI, and 0.1% cysteine-HCl). For cloning experiments as above, *Escherichia coli* DH5α was grown in LB medium containing am-

TABLE 1 - *Oligonucleotide primers used in this study.*

Name	*Sequence (5'-3')*
0445-upF-Eco	GGAATTCCGAGCAGAACCTGATTATTTCG
0445-upR-Bam	CGGGATCCCGCTTTATTCGAATTATGTTAGGAGG
0445-dnF-Bam	CGGGATCCCGTTAAAGGCGGTGAAAAGGTAC
0445-dnR-Xba	GCTCTAGAGCATCTCTTCGGGAGCAAGGAG
0716-upF-Eco	GGAATTCCGGAAGAGCTGGGAATGAAGAG
0716-upR-Bam	CGGGATCCCGCGAACGGGAGTAAAGAAAAG
0716-dnF-Bam	CGGGATCCCGATCGAATCATGCGGATCTGTC
0716-dnR-Xba	GCTCTAGAGCGAAAGATGGGAGGCAATCAAC
1431-upF-Eco	GGAATTCCATCTGGCTCTGGAGAAAGCTC
1431-upR-Bam	CGGGATCCCGTAAAGCTCATTGTTCGGGAATC
1431-dnF-Bam	CGGGATCCCGATGAACAACCTTATGGATGGC
1431-dnR-Xba	GCTCTAGAGCTCATTTTCGGCATGTGTATCC
1537-upF-Kpn	GGGGTACCCCAAGGCTGATCTCTCTATCGTG
1537-upR-Bam	CGGGATCCCGAGTGCCATCAAGAATATAAATG
1537-dnF-Bam	CGGGATCCCGATAGTTGTAGACGGCAACGCC
1537-dnR-Xba	GCTCTAGAGCGATCACCGCAAGGGAAATAG
1680-upF-Kpn	GGGGTACCCCTGTACGGCTCGAAAGAATTG
1680-upR-Bgl	GAAGATCTTCACTTATTCGATTGGCCGAAG
1680-dnF-Bgl	GAAGATCTTCGTCTGCCCTCCATCCGAAG
1680-dnR-Xba	GCTCTAGAGCGGCACCATCTTTGCGATATG
2014-upF-Kpn	GGGGTACCCCGTAGCCAAAACCTTCTACCTG
2014-upR-Bam	CGGGATCCCGTGAGAAGTGAATCTCATTGG
2014-dnF-Bam	CGGGATCCCGAGAGAGAGGATATTCGATCATGC
2014-dnR-Xba	GCTCTAGAGCACGAGTACGGCAGATTGTCC
ermFAM-F-Bam	CGGGATCCCGATAGCTTCCGCTATTGC
ermFAM-R-Bam	CGGGATCCCGAAGCTGTCAGTAGTATACC
cfxA-Fwd-Bam	CGGGATCCCGAAAATCAGTTCTTTAGCGA
cfxA-Rev-Bam	CGGGATCCCGACACAGGCGGAACTTTGATA

Undrerlined nucleotides indicate recognition sites of restriction enzymes.

picillin (100 µg/mL) and used as a host strain.
The minimum inhibitory concentrations (MICs) of antibiotics for the MFD mutants and the wild-type strain were determined using the agar dilution method (Ikeda and Yoshimura, 2002). Briefly, bacterial strains were cultured overnight in enriched BHI broth (3.7% BHI, 0.5% yeast extract, 0.1% cysteine-HCl). The overnight precultures were diluted with fresh enriched BHI broth to an optical density of 0.2 at 600 nm and grown overnight. Again, the overnight precultures were diluted as mentioned above and used to inoculate antibiotic sensitivity test plates (enriched BHI blood agar plates containing an appropriate concentration of each antibiotic). Two microliters of each bacterial suspension were spotted in triplicate on each antibiotic sensitivity test plate, and incubated anaerobically for 7 days, followed by evaluation of the MIC for each antibiotic in each strain. Each antibiotic sensitivity assay was performed at least three times.
The molecular phylogenetic tree was constructed using the ClustalW program in the GENETYX software (GENETYX Corporation, Tokyo, Japan).
The genomic DNA of the *P. gingivalis* strains ATCC 33277, W83, and TDC60 was completely sequenced, and the gene annotation tables are available on the Kyoto Encyclopedia of Genes and Genomes (KEGG) website (http://www.kegg.jp/kegg/). It is well-known that TDEP shows structural similarity to the type I secretion system (T1SS) (Delepelaire, 2004), which transports toxins and macromolecule-degrading enzymes. Analysis of the genome sequence of *P. gingivalis* ATCC 33277 revealed that this bacterium possesses six gene clusters encoding TDEPs and one T1SS gene cluster. This T1SS, encoded by the genes PGN_2040 to PGN_2038, was strongly expected to secrete alkaline protease because the T1SS operon appeared to include an adjacent gene, PGN_2041 that was annotated as an alkaline protease gene, similar to the gene organization of the hemolysin operon (*hylCABD*) in *E. coli* (Welch and Pellett, 1988), a typical T1SS. Therefore, the present study focused on the remaining gene clusters, I to VI.
Figure 1 shows gene arrangement in gene clusters encoding TDEPs in *Porphyromonas gingivalis* ATCC 33277. Each gene cluster was found to be composed of three components; OMP, CMP, and MFP. However, some variation was observed among the six gene clusters. Clusters I, II, IV, and V contained more than two CMPs, and cluster V lacked an OMP. A similar variation in the genetic organization of efflux pump operons has been found in other bacteria (Schweizer, 2003; Matsuo *et al*., 2013). In the absence of an OMP in an efflux pump operon, it is assumed that an OMP from another efflux pump or an OMP encoded by a gene not linked to the efflux pump operons is recruited to form a channel to extrude toxic compounds. A typical model has been reported in *E. coli*; neither the *acrAB* nor the *macAB* drug efflux operon contains an OMP gene, and they share a single outer membrane pore protein, TolC, instead (Sun *et al.*, 2014; Lu and Zgurskaya, 2012). These drug efflux systems, AcrAB-TolC and MacAB-TolC,

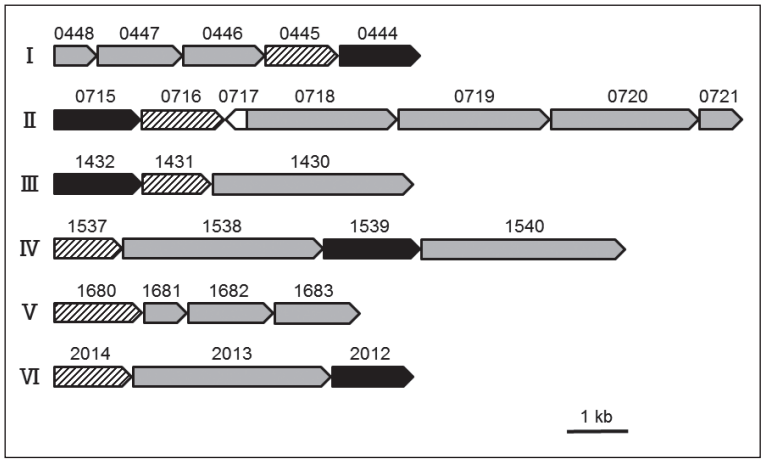

FIGURE 1 - *Gene arrangement in gene clusters encoding TDEPs in Porphyromonas gingivalis ATCC 33277. Black box, OMP gene; hatched box, MFP gene; gray box, CMP gene. The number beside each gene indicates the PGN No. in the P. gingivalis ATCC 33277 genome database.*

are representative of two types of TDEPs, the RND-family pumps and the ABC-family pumps with OMP and MFP, respectively. The AcrAB-TolC system is involved in efflux of various toxic substrates, including various antibiotics, disinfectants, and bile salts, driven by a proton motive force (PMF), whereas the MacAB-TolC system is responsible for macrolide-specific efflux, driven by ATP (Sun *et al*., 2014; Lu and Zgurskaya, 2012). In *P. gingivalis* ATCC 33277, the gene annotation table available on KEGG database indicates that the gene clusters I, II, and V encode ABC-family pumps, whereas the clusters III, IV, and VI encode RND-family pumps. Based on the amino acid sequences of the MFPs encoded by these gene clusters, a molecular phylogenetic tree was constructed for MFPs of *P. gingivalis* strains using the ClustalW program (Figure 2). As expected, the MFPs of RND-family efflux pumps and the MFPs of ABC-family efflux pumps formed separate clusters. In *P. gingivalis* strain W83, gene cluster IV could not be found. However, all of the other gene clusters were conserved in *P. gingivalis* W83 and TDC60 (data not shown).

Among the six gene clusters encoding *P. gingivalis* TDEPs, several encode more than two CMPs, and one of the clusters does not encode an OMP. However, all six gene clusters have a single MFP gene (Figure 1). Therefore, we constructed MFP gene (PGN_0445, PGN_0716, PGN_1431, PGN_1537, PGN_1680, and PGN_2014)-deletion mutants to examine the effect of each drug efflux pump on the antibiotic sensitivity of *P. gingivalis* ATCC 33277. Each MFP gene was replaced with an erythromycin resistance gene cassette by double-crossover homologous recombination. These mutants were designated as MFD445, MFD716, MFD1431, MFD1537, MFD1680, and MFD2014, respectively.

The MICs for the MFD mutants of five antibiotics were determined using the agar-dilution method (Ikeda and Yoshimura, 2002). All of the MFD mutants showed the same level of growth as the wild-type strain on antibiotic sensitivity test plates without antibiotics after 7 days of incubation. Table 2 shows the antibiotic sensitivity profiles of the mutants. Tetracycline sensitivity increased in all MFD mutants compared to the wild-type strain. In contrast, no MFD mutants showed increased sensitivity to chloramphenicol (data not shown). Both MFD1431 and MFD1680 were more sensitive to all four antibiotics than the wild-type strain, suggesting that these two TDEPs might be major functional TDEPs of *P. gingivalis* ATCC 33277, similar to the *E. coli* AcrAB-TolC system. Previously, the PGN_1431 gene was designated *xepA* by Ikeda and Yoshimura (2002). Our results for the antibiotic sensitivity profile of MFD1431 are almost in concordance with those for the *xepA*-deletion mutant. However, MFD1431 was more susceptible to ampicillin than the wild-type, whereas the *xepA* mutant was not. The discrepancy may

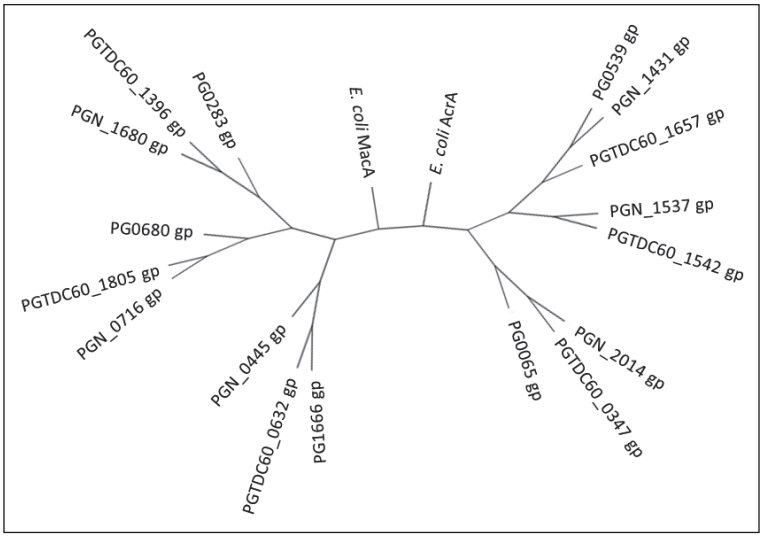

FIGURE 2 - *Molecular phylogenetic tree of MFP gene products constructed using the ClustalW program based on their amino acid sequences in the Porphyromonas gingivalis strains for which the entire genome sequences are available, ATCC 33277, W83, and TDC60. Escherichia coli acrA and macA gene products are also included as representatives of MFPs of RND-family pumps and ABC-family pumps, respectively. No ortholog of PGN_1537 is present in W83.*

TABLE 2 - *MICs of four antibiotics in P. gingivalis ATCC 33277 and its MFD mutants.*

	MIC (μg/mL)			
	Amp	*Rif*	*Tet*	*Nor*
Wild-type	0.1	0.0075	0.24	4
MFD445	0.1	0.0075	0.12	4
MFD716	0.1	0.0075	0.12	4
MFD1431	0.05	0.0038	0.12	2
MFD1537	0.1	0.0075	0.12	2
MFD1680	0.05	0.0038	0.12	2
MFD2014	0.1	0.0075	0.12	4

Amp, ampicillin; Rif, rifampicin; Tet, tetracycline; Nor, norfloxacin. None of the MFD mutants exhibited increased sensitivity to chloramphenicol.

have been caused by differences in the culture conditions. Of the present results, the most interesting is the drug efflux system encoded by gene cluster V. This system is presumed to belong to the ABC family of pumps because the CMPs encoded by this system are annotated as an ATP transporter ATP-binding protein (gene product of PGN_1681 [PGN_1681 gp]) and ABC transporter permease proteins (PGN_1682 gp and PGN_1683 gp). However, the MFD1680 mutant was more susceptible to multiple antibiotics, indicating that the efflux pump encoded by gene cluster V may be involved in efflux of various toxic substrates, similar to the RND-family pumps.

As mentioned above, both MFD1431 and MFD1680 commonly showed increased sensitivity to multiple antibiotics, although the MIC decreased twofold for all antibiotics used. Notably, Matsuo *et al*. (2013) and Bina *et al*. (2008) reported that multiple-pump mutants were more sensitive to antibiotics and detergents than single-pump mutants in *Vibrio parahaemolyticus* and *Vibrio cholerae*, which can be explained by functional redundancy among several pumps. In *P. gingivalis*, PGN_1431 gp and PGN1680 gp may be functionally redundant. To test this hypothesis, we obtained two independent PGN_1431-deletion mutants of MFD1680, designated MFDD3 and MFDD8, which were subsequently subjected to an antibiotic sensitivity assay. Unexpectedly, these double knockout mutants showed no significant differences in susceptibility to the antibiotics used above, compared to MFD1431 and MFD1680. One plausible explanation for this would be that in the MFDD3 and MFDD8 mutants, MFPs from other efflux pumps partially compensated for the lack of the MFPs encoded by PGN_1431 and PGN_1680. If this is the case, further multiple mutations in genes encoding other TDEPs could increase antibiotic sensitivity. Alternatively, in MFDD3 and MFDD8 mutants, other types of efflux systems, such as MATE-family pumps (Kuroda and Tsuchiya, 2009), might have been activated. In the gene annotation table for *P. gingivalis* ATCC 33277, we identified five MATE-family pump genes. In addition, we found orthologs of all of the MATE-family genes in both the *P. gingivalis* W83 and TDC60 genomes. The activities and substrate specificities of these MATE-family pumps remain to be elucidated.

In the present study, we constructed six TDEP mutants of *P. gingivalis* ATCC 33277. Compared to the wild-type strain, the MICs of four antibiotics were reduced in these mutants, suggesting that the activity of drug efflux systems may affect antibiotic sensitivity in *P. gingivalis*.

Currently, treatment with systemic antibiotics is one of the most common therapies for periodontitis. Recently, Keestra *et al*. (2014) reported that systemic antibiotics in combination with scaling and root planing (SRP) result in additional clinical benefits compared to only SRP (Keestra *et al*., 2014). The most common systemic antibiotics for periodontal therapy are penicillins (amoxicillin), tetracyclines (tetracycline, minocycline, doxycycline), macrolides (clindamycin, azithromycin), quinolones (ciprofloxacin, moxifloxacin, levofloxacin) and metronidazole. It should be noted that reduced antibiotic susceptibilities of clinical isolates of *P. gingivalis* have been reported for amoxicillin, ciprofloxacin, clindamycin, doxycycline and metronidazole (Ardila *et al*., 2010; Eick *et al*., 1999; Japoni *et al.*, 2011; Veloo *et al*., 2012). Previously, Eick *et al.* reported amoxicillin resistance based on β-lactamase production in *P. gingivalis* (Eick *et al*., 1999). However, mechanisms for the reduced antibiotic sensitivities are almost unknown. The present study suggested multiple antibiotics as potential substrates for TDEPs. Although the MIC differences between wild-type and TDEP mutants were only twofold, a further increase of MIC in the presence of antibiotics could be possible as

other bacteria like *Pseudomonas aeruginosa*, in which overexpression mutations in TDEP regulatory genes were observed as a consequence of exposure to antimicrobials (Schweizer, 2003). In *P. gingivalis* - closely related *Bacteroides fragilis* - 16 RND-family efflux pumps have been identified in its genome (Wexler, 2012). Interestingly, these efflux pumps were able to pump out a variety of substrates and the expression of these pumps was increased in the presence of antimicrobial and antiseptic agents. In *P. gingivalis*, the activity of the drug efflux pumps could be elevated, resulting in reduced antibiotic sensitivity.

Finally, in addition to antibiotic susceptibility, an increasing number of reports have indicated that drug efflux pumps are associated with virulence in some gram-negative species (Sun *et al*., 2014; Webber *et al*., 2009). Interestingly, mutation of a single drug efflux pump altered the expression levels of hundreds of genes in *Salmonella enterica* (Webber *et al*., 2009). Therefore, we could not exclude the possibility that our TDEP mutants could change other cellular properties affecting antibiotic sensitivity, for example the outer membrane permeability of antibiotics. Thus, more detailed studies are warranted to clarify the roles of drug efflux pumps in antibiotic sensitivity as well as virulence and physiological states in *P. gingivalis*.

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